

NANO EXPRESS

Open Access

Earthworm extracts utilized in the green synthesis of gold nanoparticles capable of reinforcing the anticoagulant activities of heparin

Hee Kyeong Kim¹, Myung-Jin Choi², Song-Hyun Cha², Yean Kyoung Koo³, Sang Hui Jun¹, Seonho Cho² and Youmie Park^{1,2*}

Abstract

Gold nanoparticles were obtained using a green synthesis approach with aqueous earthworm extracts without any additional reducing or capping agents. The gold nanoparticles were characterized using UV-visible spectrophotometry, high-resolution transmission electron microscopy, atomic force microscopy, field emission scanning electron microscopy, X-ray diffraction, Fourier transform infrared spectroscopy, and inductively coupled plasma mass spectrometry. The anticoagulant activity of the gold nanoparticles was assessed using the activated partial thromboplastin time and was mildly enhanced by combining the gold nanoparticles with heparin. In addition to the generation of spherical nanoparticles with an average diameter of 6.13 ± 2.13 nm, cubic and block-shaped nanoparticles with an average aspect ratio, defined as the length divided by width, of 1.47 were also observed.

Keywords: Green synthesis; Earthworm extracts; Invertebrate natural products; Gold nanoparticles; Anticoagulant activity

Background

Recent advances in nanotechnology have enabled the exploration of nanomaterials for diverse applications. Among the variety of nanomaterials, gold nanoparticles (AuNPs) are of considerable interest due to their versatility and potential uses in chemistry, biology, medicine, and pharmaceuticals. AuNPs possess numerous advantages, such as low cytotoxicity, facile modification of their surfaces, straightforward synthetic processes, and excellent biocompatibility [1,2]. Currently, research interest in gold nanomedicine is rapidly expanding. In 2010, approximately 14% of all publications on nanomedicine were directly related to gold nanomedicine [3].

The common approach for synthesizing AuNPs employs sodium citrate and/or sodium borohydride as reducing agents to convert gold salts into AuNPs. The emergence of sustainability initiatives has increased the use of biological

entities as reducing agents in AuNP synthesis (i.e., green synthesis) to replace toxic chemicals. Many authors have extensively reported the green synthesis of AuNPs using diverse biological entities. These green synthetic processes are rapid, eco-friendly, and cost-effective, and they can easily be scaled up [4-8]. Examples of these diverse biological entities include plant extracts, polysaccharides, bacteria, fungi, yeasts, DNA, RNA, proteins, and polypeptides. We used aqueous earthworm (*Eisenia andrei*) extracts as a reducing agent for the green synthesis of AuNPs (EW-AuNPs). Earthworm extracts reportedly have anticoagulant, fibrinolytic, and antithrombotic activities [9-14]. Trisina and co-workers reported that the protein extracts from *Lumbricus rubellus* are responsible for antithrombotic and thrombolytic activities [14]. In addition to proteins, glycosaminoglycans (chondroitin/dermatan sulfates and heparan sulfate) are also present in earthworm (*E. andrei*) extracts [15].

EW-AuNPs were characterized using UV-visible spectrophotometry, high-resolution transmission electron microscopy (HR-TEM), atomic force microscopy (AFM), field emission scanning electron microscopy (FE-SEM), X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FT-IR), and inductively coupled plasma mass

* Correspondence: youmiepark@inje.ac.kr

¹College of Pharmacy, Inje University, 607 Obang-dong, Gimhae, Gyeongnam 621-749, Republic of Korea

²National Creative Research Initiatives (NCRI) Center for Isogeometric Optimal Design, Seoul National University, 1 Gwanak-ro, Gwanak-gu, Seoul 151-744, Republic of Korea

Full list of author information is available at the end of the article

spectrometry (ICP-MS). As previously mentioned, anticoagulant activity is reportedly among the major biological activities of earthworm extracts; therefore, we assessed the anticoagulant activities of EW-AuNPs both alone and in combination with heparin.

Methods

Hydrochloroauric acid trihydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$) and Minisart® syringe filters (0.45 μm ; Sartorius AG, Goettingen, Germany) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Earthworm (*E. andrei*) powders were obtained from a local supplier (Hwasun, Cheollanam-Do, Republic of Korea). Heparin sodium injection was obtained from JW Pharmaceutical (Seoul, Republic of Korea). All other reagents were of analytical grade. We previously reported the green synthesis of AuNPs using aqueous earthworm (*E. andrei*) extracts, the reaction process was optimized, and HR-TEM images of the AuNPs were obtained [16]. This procedure, with a minor modification, was utilized in this study. The earthworm powder (150 mg) was dispersed in deionized water (50 mL) and sonicated for 30 min. The insoluble pellet was removed after centrifugation at $5,067 \times g$ for 10 min (Eppendorf 5424R centrifuge, Eppendorf AG, Hamburg, Germany). The supernatant was subsequently filtered through filter paper and a Minisart® filter (0.45 μm) and then freeze-dried. The freeze-dried material was used to synthesize the EW-AuNPs according to the following procedures: the earthworm extract (500 μL , 0.3% in deionized water) was mixed with $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ (500 μL , 0.6 mM in deionized water), and the mixture was incubated in an 80°C oven for 11 h. The reaction yield was measured by detecting the concentration of unreacted Au^{3+} via ICP-MS, which was conducted using an ELAN 6100 instrument (PerkinElmer SCIEX, Waltham, MA, USA). The samples containing unreacted Au^{3+} were prepared either by ultracentrifugation or by filtration. Ultracentrifugation was performed in an Eppendorf 5424R centrifuge at $21,130 \times g$ for 1 h at 18°C. Under this ultracentrifugation condition, AuNPs remained as a wine-red pellet, and the color of the supernatant turned colorless. The supernatant containing the unreacted Au^{3+} was then pooled and analyzed via ICP-MS. The EW-AuNP solution was filtered through a syringe equipped with a Minisart® filter (0.45 μm). The colorless filtrate was also analyzed via ICP-MS. ICP-MS analysis was performed in triplicate to obtain an average yield. A Shimadzu UV-1800 spectrophotometer was used to acquire the UV-visible spectra (Shimadzu Corporation, Kyoto, Japan). A JEOL JEM-3010 TEM (JEOL Ltd., Tokyo, Japan) operating at 300 kV with samples on a carbon-coated copper grid (carbon type-B, 300 mesh, Ted Pella Inc., Redding, CA, USA) was used to obtain the HR-TEM. The AFM images were acquired using a Dimension® Icon® (Bruker Nano, Inc., Santa Barbara, CA, USA) with an

RTESP probe (MPP-11100-10, premium high-resolution tapping-mode silicon probe, Bruker Nano, Inc., Santa Barbara, CA, USA) in tapping mode. The mica (grade V-1, 25 mm \times 25 mm, 0.15-mm thick) was acquired from the SPI Supplies Division of Structure Probe, Inc. (West Chester, PA, USA) and was used for the sample deposition. FE-SEM images were obtained using a JSM-7100 F with an accelerating voltage of 15 kV (JEOL Ltd., Tokyo, Japan). The samples were lyophilized with a FD5505

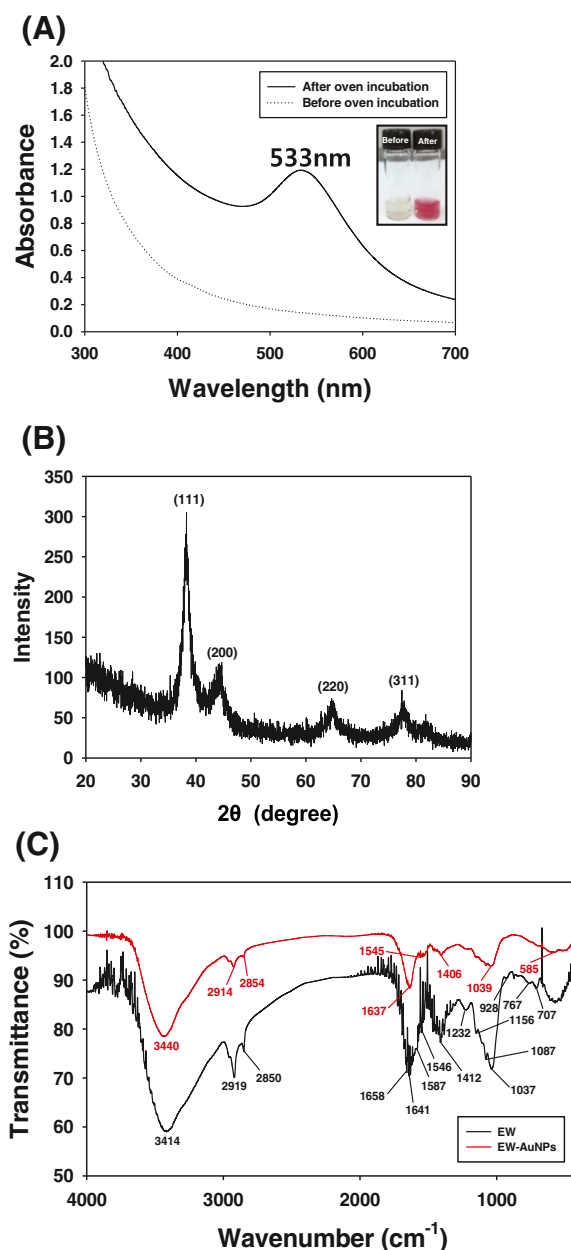


Figure 1 UV-visible spectra, XRD analysis, and FT-IR spectra of EW-AuNPs. **(A)** UV-visible spectra before and after the oven incubation. The inset depicts the color change of the AuNP solution. **(B)** XRD analysis of the EW-AuNPs. **(C)** FT-IR spectra of the EW and EW-AuNPs.

freeze drier (Il Shin Bio, Seoul, Republic of Korea). The FT-IR spectra were acquired with a KBr pellet of the freeze-dried samples using a Nicolet 6700 spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) over a range of $400 \sim 4,000 \text{ cm}^{-1}$. XRD analysis was performed using a Bruker D8 Discover high-resolution X-ray diffractometer (Bruker, Karlsruhe, Germany) with $\text{CuK}\alpha$ radiation ($\lambda = 0.154056 \text{ nm}$) over the range of $20^\circ \sim 90^\circ$ (2θ scale). A tenfold AuNP concentrate was processed under an N_2 atmosphere to assess the activated partial thromboplastin time (aPTT) using a procedure adapted from our previous report [17].

Results and discussion

Green synthesis and yield of EW-AuNPs

As depicted in Figure 1A, the wine-red color of the EW-AuNPs after incubation in an oven confirmed the successful synthesis of the AuNPs. The surface plasmon resonance band of AuNPs was observed at 533 nm. ICP-MS is an

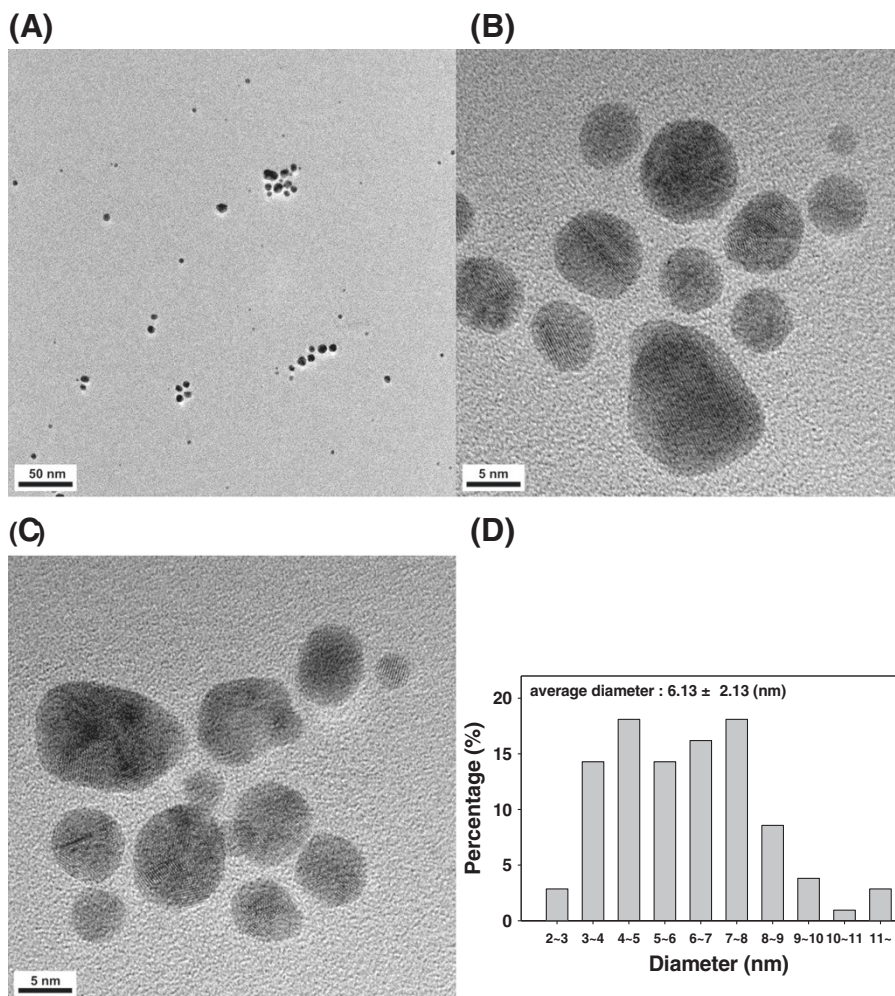
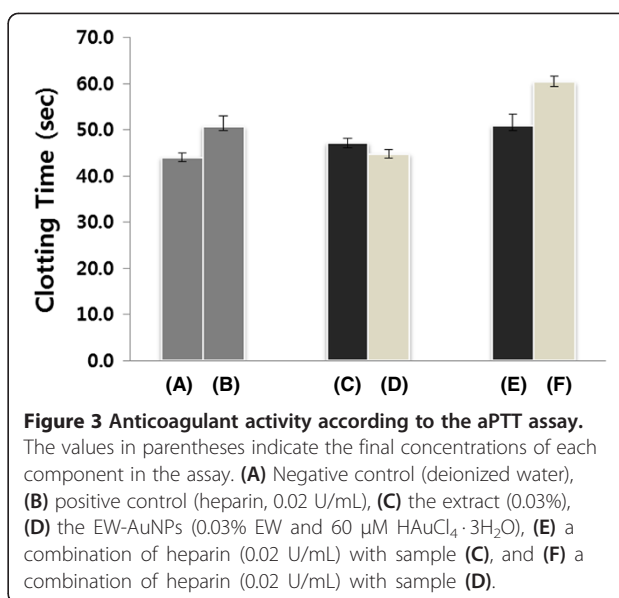


Figure 2 HR-TEM images of the EW-AuNPs. The scale bar represents (A) 50 nm, (B) 5 nm, and (C) 5 nm. (D) Size histogram.

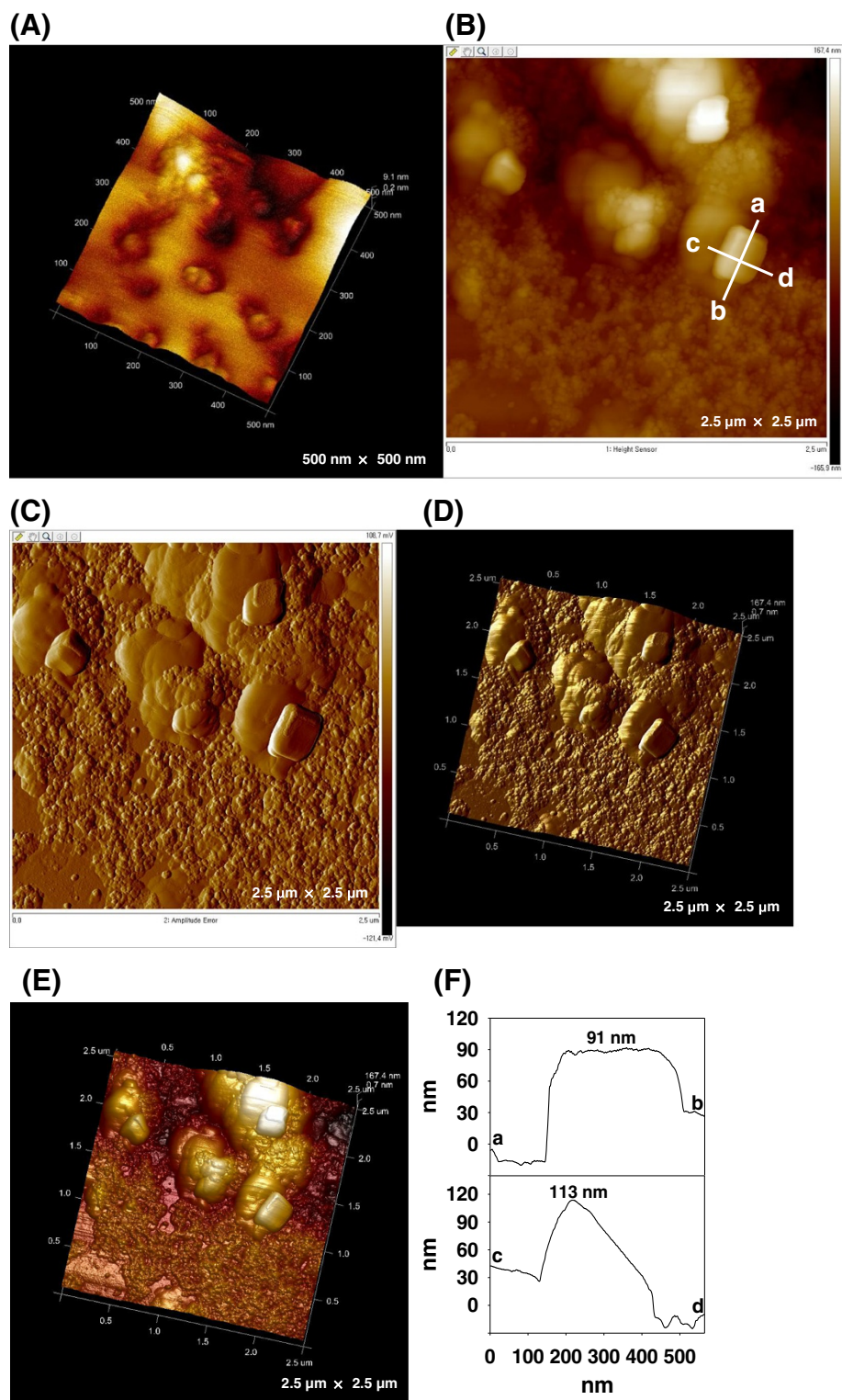


Figure 4 AFM images of the EW-AuNPs. (A) 3-D height image (500 nm × 500 nm), (B) 2-D height image (2.5 μm × 2.5 μm), (C) 2-D amplitude error image (2.5 μm × 2.5 μm), (D) 3-D amplitude error image (2.5 μm × 2.5 μm), (E) 3-D height image (2.5 μm × 2.5 μm), and (F) cross-sectional analysis of both the length (line a-b) and the width (line c-d) from B.

excellent detection tool for measuring the concentration of unreacted Au^{3+} at the ppt level. The concentration of the EW-AuNPs solution was measured by ICP-MS as 95,192.2 parts per billion (ppb) which was the initial Au^{3+} concentration used for the synthesis. The concentrations of the unreacted Au^{3+} were measured by ICP-MS as 8,455.6 and 7,151.1 ppb with the ultracentrifugation and filtration methods, respectively. Thus, the ultracentrifugation method obtained a yield of 91.1%, and the filtration method obtained a yield of 92.5%. The characteristic wine-red color of the EW-AuNPs disappeared after ultracentrifugation or filtration, indicating that the AuNPs were successfully separated from the unreacted Au^{3+} .

XRD analysis

The crystalline nature of the EW-AuNPs was determined via XRD analysis, as shown in Figure 1B. The diffraction peaks at 38.3° , 44.7° , 64.7° , and 77.4° corresponded to the (111), (200), (220), and (311) planes of crystalline Au, respectively, indicating a face-centered cubic structure.

FT-IR spectra

As shown in Figure 1C, in the earthworm sample, the O-H stretching vibration appeared at $3,414\text{ cm}^{-1}$ as an

intense and broad band. The two bands at 2,919 and $2,850\text{ cm}^{-1}$ were identified as the methylene vibrations of the hydrocarbons from the proteins/peptides [18]. The carbonyl ($\text{C}=\text{O}$) stretching vibration at $1,658\text{ cm}^{-1}$ from the amide functional groups also indicated the presence of proteins/peptides [18,19]. The band at $1,587\text{ cm}^{-1}$ resulted from the N-H bending vibration of the amide functional groups. The COO^- stretching vibration appeared at $1,412\text{ cm}^{-1}$. The bands from the earthworm sample suggested that proteins/peptides were the major compounds present in the sample. After synthesis of the EW-AuNPs, these bands shifted from $3,414$ to $3,440\text{ cm}^{-1}$, from 2,919 to $2,914\text{ cm}^{-1}$, from 2,850 to $2,854\text{ cm}^{-1}$, from 1,658 to $1,637\text{ cm}^{-1}$, and from 1,412 to $1,406\text{ cm}^{-1}$. Based on these shifts, the proteins/peptides in the extract are likely responsible for the reduction of Au^{3+} to generate the AuNPs.

HR-TEM images and size histogram

The EW-AuNPs were primarily spherical with an average diameter of $6.13 \pm 2.13\text{ nm}$ (Figure 2). A collection of 105 discrete AuNPs were randomly selected from the HR-TEM images to measure the average diameter. The two most abundant diameters were $4 \sim 5$ and $7 \sim 8\text{ nm}$,

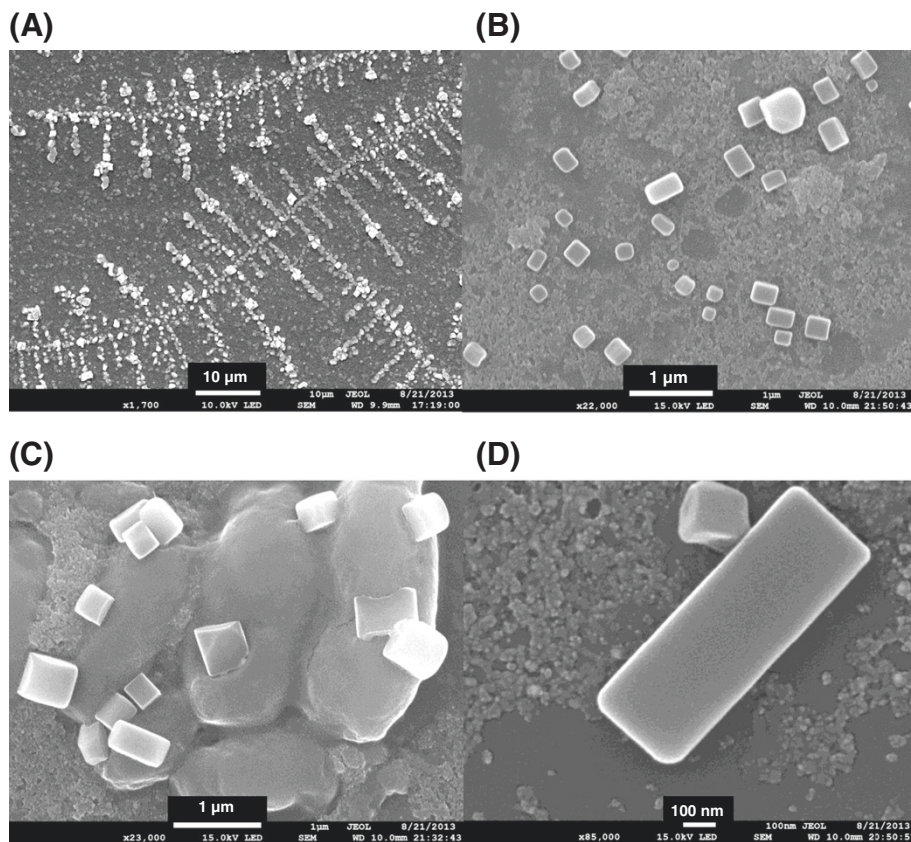


Figure 5 FE-SEM images of the EW-AuNPs. The scale bar represents (A) 10 μm , (B) 1 μm , (C) 1 μm , and (D) 100 nm.

which accounted for 19% of the total (Figure 2D). Clear lattice fringes further confirmed the crystalline structure of the EW-AuNPs (Figure 2B,C). We previously obtained spherical EW-AuNPs with the diameter of 6.70 ± 2.69 nm using a green synthesis route with different reaction conditions [16].

Anticoagulant activity via aPTT assay

The EW-AuNPs reinforced or enhanced the anticoagulant activity of heparin by aPTT assay when the combination of EW-AuNPs and heparin was used for treatment (Figure 3). The clotting times of the negative (deionized water) and positive (heparin) controls were 44.1 and 50.8 s, respectively (Figure 3 parts A and B). No significant anticoagulant activities were noted in the extract (47.2 s, Figure 3 part C), the EW-AuNPs (44.8 s, Figure 3 part D), or in heparin combined with the extract (50.9 s, Figure 3 part E). However, when heparin and the EW-AuNPs were combined, the clotting time was extended to 60.4 s (Figure 3 part F), which corresponds to an increase of 118.9% and 134.8% over the clotting times of the same concentrations of the positive control (heparin) and the EW-AuNPs, respectively.

AFM images

As depicted in Figure 4A, the obtained AuNPs were primarily spherical. This result is consistent with the HR-TEM images presented in Figure 2. Following an ultracentrifugation/resuspension process, the pellets (EW-AuNPs) were redispersed in deionized water and examined via AFM. The 2-D and 3-D images demonstrated that cubic and block-shaped AuNPs were also present as minor components (Figure 4B,C,D,E). Cross-sectional analysis further confirmed the block shape of the AuNPs (Figure 4F).

FE-SEM images

When we imaged the cubic and block-shaped AuNPs via FE-SEM, these shapes appeared in a line that resembled fish bones (Figure 5A). A more detailed examination revealed cubic and block-shaped anisotropic particles. The width varied from 0.11 to 0.33 μm , and the length varied from 0.13 to 0.93 μm (Figure 5B). The aspect ratio is defined as the length divided by the width. The average aspect ratio was 1.47, with values ranging between 1.0 and 2.8. The largest observed block had a width of 0.33 μm , a length of 0.93 μm , and a maximum aspect ratio of 2.8 (Figure 5D). Murphy and co-workers reported that surfactants such as cetyltrimethylammonium bromide or small ions act as structure-directing agents in the formation of anisotropic nanostructures [20]. We hypothesize that the structure-directing agents in the extracts likely induced the formation of anisotropic shapes during synthesis. We previously reported the presence of glycosaminoglycans in these earthworm extracts [15]. Glycosaminoglycans are

water-soluble compounds with large negative charges that can act as structure-directing agents. Based on the interpretation of the FT-IR spectra, proteins/peptides are the likely other candidates.

Conclusions

We report the green synthesis of AuNPs using aqueous earthworm extracts as reducing agents to convert Au^{3+} to AuNPs and the characterization of these AuNPs. The reactions occurred in water without the use of any other toxic chemicals; thus, the resulting AuNPs were available for subsequent biological tests. Anisotropic NPs were observed in addition to the spherical NPs. We are unable to explain how the anisotropic NPs were generated, and this topic will be explored in future work. From the FT-IR spectra, we could conclude that the proteins/peptides in the extract were involved in the reduction of Au^{3+} and in the stabilization of the EW-AuNPs. In addition, the anticoagulant activity of heparin was reinforced when combined with the EW-AuNPs, which suggests that AuNPs are involved in the blood coagulation cascade. The current study demonstrates that the newly prepared AuNPs are promising candidates for novel gold nanomedicines.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

HKK and SHJ performed the green synthesis of the EW-AuNPs. MJC, SHC, and YP characterized the EW-AuNPs. YKK performed the aPTT assay. SC and YP supervised the entire process and drafted the manuscript. All authors read and approved the final manuscript.

Acknowledgements

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government: the Ministry of Education (NRF-2012R1A1A2042224) and the Ministry of Science, ICT & Future Planning (NRF-2010-18282).

Author details

¹College of Pharmacy, Inje University, 607 Obang-dong, Gimhae, Gyeongnam 621-749, Republic of Korea. ²National Creative Research Initiatives (NCRI) Center for Isogeometric Optimal Design, Seoul National University, 1 Gwanak-ro, Gwanak-gu, Seoul 151-744, Republic of Korea. ³College of Pharmacy, Seoul National University, 1 Gwanak-ro, Gwanak-gu, Seoul 151-742, Republic of Korea.

Received: 15 October 2013 Accepted: 11 December 2013

Published: 26 December 2013

References

1. Sperling RA, Rivera Gil P, Zhang F, Zanella M, Parak WJ: **Biological applications of gold nanoparticles.** *Chem Soc Rev* 2008, **37**:1896–1908.
2. Yeh YC, Creran B, Rotello VM: **Gold nanoparticles: preparation, properties, and applications in bionanotechnology.** *Nanoscale* 2012, **4**:1871–1880.
3. Dreaden EC, Alkilany AM, Huang X, Murphy CJ, El-Sayed MA: **The golden age: gold nanoparticles for biomedicine.** *Chem Soc Rev* 2012, **41**:2740–2779.
4. Park Y, Hong YN, Weyers A, Kim YS, Linhardt RJ: **Polysaccharides and phytochemicals: a natural reservoir for the green synthesis of gold and silver nanoparticles.** *IET Nanobiotechnol* 2011, **5**:69–78.
5. Kharisova OV, Dias HV, Kharisov BI, Pérez BO, Pérez VM: **The greener synthesis of nanoparticles.** *Trends Biotechnol* 2013, **31**:240–248.

6. Faramarzi MA, Sadighi A: **Insights into biogenic and chemical production of inorganic nanomaterials and nanostructures.** *Adv Colloid Interface Sci* 2013, **189**–190:1–20.
7. Mittal AK, Chisti Y, Banerjee UC: **Synthesis of metallic nanoparticles using plant extracts.** *Biotechnol Adv* 2013, **31**:346–356.
8. Panda T, Deepa K: **Biosynthesis of gold nanoparticles.** *J Nanosci Nanotechnol* 2011, **11**:10279–10294.
9. Hahn BS, Jo YY, Yang KY, Wu SJ, Pyo MK, Yun-Choi HS, Kim YS: **Evaluation of the *in vivo* antithrombotic, anticoagulant and fibrinolytic activities of *Lumbricus rubellus* earthworm powder.** *Arch Pharm Res* 1997, **20**:17–23.
10. Kim YS, Pyo MK, Park KM, Hahn BS, Yang KY, Yun-Choi HS: **Dose dependency of earthworm powder on antithrombotic and fibrinolytic effects.** *Arch Pharm Res* 1998, **21**:374–377.
11. Lee CK, Shin JS, Kim BS, Cho IH, Kim YS, Lee EB: **Antithrombotic effects by oral administration of novel proteinase fraction from earthworm *Eisenia andrei* on venous thrombosis model in rats.** *Arch Pharm Res* 2007, **30**:475–480.
12. Hrzenjak T, Popović M, Božić T, Grdisa M, Kobrehel D, Tiska-Rudman L: **Fibrinolytic and anticoagulative activities from the earthworm *Eisenia foetida*.** *Comp Biochem Physiol B Biochem Mol Biol* 1998, **119**:825–832.
13. Cooper EL, Hrzenjak TM, Grdisa M: **Alternative sources of fibrinolytic, anticoagulative, antimicrobial and anticancer molecules.** *Int J Immunopathol Pharmacol* 2004, **17**:237–244.
14. Trisina J, Sunardi F, Suhartono MT, Tjandrawinata RR: **DLBS1033, a protein extract from *Lumbricus rubellus*, possesses antithrombotic and thrombolytic activities.** *J Biomed Biotechnol* 2011, **2011**:519652.
15. Im AR, Park Y, Sim JS, Zhang Z, Liu Z, Linhardt RJ, Kim YS: **Glycosaminoglycans from earthworms (*Eisenia andrei*).** *Glycoconj J* 2010, **27**:249–257.
16. Han L, Kim YS, Cho S, Park Y: **Invertebrate water extracts as biocompatible reducing agents for the green synthesis of gold and silver nanoparticles.** *Nat Prod Commun* 2013, **8**:1149–1152.
17. Kim HS, Jun SH, Koo YK, Cho S, Park Y: **Green synthesis and nanotopography of heparin-reduced gold nanoparticles with enhanced anticoagulant activity.** *J Nanosci Nanotechnol* 2013, **13**:2068–2076.
18. Zhang YX, Zheng J, Gao G, Kong YF, Zhi X, Wang K, Zhang XQ, Cui da X: **Biosynthesis of gold nanoparticles using chloroplasts.** *Int J Nanomedicine* 2011, **6**:2899–2906.
19. Xia Y, Wan J, Gu Q: **Silk fibroin fibers supported with high density of gold nanoparticles: fabrication and application as catalyst.** *Gold Bull* 2011, **44**:171–176.
20. Murphy CJ, Sau TK, Gole AM, Orendorff CJ, Gao J, Gou L, Hunyadi SE, Li T: **Anisotropic metal nanoparticles: synthesis, assembly, and optical applications.** *J Phys Chem B* 2005, **109**:13857–13870.

doi:10.1186/1556-276X-8-542

Cite this article as: Kim et al.: Earthworm extracts utilized in the green synthesis of gold nanoparticles capable of reinforcing the anticoagulant activities of heparin. *Nanoscale Research Letters* 2013 **8**:542.

Submit your manuscript to a SpringerOpen[®] journal and benefit from:

- Convenient online submission
- Rigorous peer review
- Immediate publication on acceptance
- Open access: articles freely available online
- High visibility within the field
- Retaining the copyright to your article

Submit your next manuscript at ► springeropen.com